1 427 253

PATENT SPECIFICATION (11) 1 427 253

(21) Application No. 21736/73 (22) Filed 7 May 1973
(44) Complete Specification published 10 March 1976
(51) INT CL² A61K 37/78 // (A61K 37/78, 31/355)
(52) Index at acceptance A5B 248 24Y 26Y 364 36Y 382 38Y 779

(19)

(54) ANTILIPEMIC COMPOSITION CONTAINING UNSAPONIFIABLE MATTER OF SOYBEAN OIL

(71) We, TAKASHI KANEDA of No. 14—1—44, Sanjo-machi, Sendai-shi, Miyagi-ken, Japan and TOSHIKAZU TABATA of No. 748, Kikuna-machi, Kohoku-ku, Yokohama-shi, Kanagawa-ken, Japan, both citizens of Japan, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

This invention relates to an antilipemic composition i.e. one which acts to lower the level of fats or lipids in blood serum.

It has been established in the prior art that certain plant sterols (also termed phytosterols), including β-sitosterol and stigmasterol, are effective for lowering the cholesterol level in blood serum[1]. A number of such reports on the antilipemic activity of plant sterols have been published. An antilipemic agent containing 20% by weight of β-sitosterol suspended in a 4% alcohol solution has recently been put on the market by E. Lilly Co. At the present time there exists no known method by which plant sterols other than β-sitosterol, e.g., stigmasterol and campesterol, can be extracted and separated, in pure form, from vegetable oils. The use of β-sitosterol as an antilepemic agent requires that dosages be administered in disadvantageously large amounts, i.e., 20 to 30 g of β-sitosterol per day, because of the body's low absorption of β-sitosterol[1], thus inviting liver or kidney troubles over long periods of administration. Additionally, where the β-sitosterol containing antilipemic agent is administered over a long period of time, a rebound phenomenon in the cholesterol value is observed during the administration period.

It has now been discovered that when an antilipemic composition which contains as the active ingredient a specific nonsaponifiable fraction of soybean oil is used, the dosing amount of active ingredient can be reduced to less than 1/10th (1.2—1.8 g per day) that required for β-sitosterol, thus reducing potential kidney and liver problems.

Thus, the present invention provides an antilipemic composition comprising a nonsaponifiable fraction of soybean oil and an orally administerable carrier, said nonsaponifiable fraction containing about 45% by weight of plant sterols, including campesterol, stigmasterol and β-sistosterol, and about 20% by weight of tocopherols.

Blood and urine examinations seem to show that the newly discovered antilipemic composition produces no adverse secondary effects even when administered over a long period of time. Furthermore, it has been confirmed through laboratory and clinical studies that the plant sterols become substituted for a part of the animal sterols (cholesterols) in the liver and blood. The composition containing the nonsaponifiable fraction of soybean oil should be administered in doses containing no more than 600 to 900 mg of phytosterols to attain a satisfactory clinical result. It has been discovered that where the present nonsaponifiable fraction of soybean oil is administered to human beings in total dosages of 1200 to 1800 mg a day, the total cholesterol level in the blood serum is lowered on the average of 10% to 15%, but the true lowering of the cholesterol level is thought to be about 20% to 25% since about 10% of the cholesterols in the serum are replaced by phytosterols.

From the foregoing, it will be understood that the antilipemic composition of the present invention offers the advantage that the dosage can be significantly reduced as compared with

[1]/See, for example, *Steroid Biochemistry and Pharmacology* M. H. Briggs and J. Brotherton — Academic Press (London: 1970), p. 183.

the prior art β-sitosterol-containing agent which requires total dosages of 20 to 30 g per day.

The unsaponifiable fraction of soybean oil which is employed as an active component of the antilipemic composition of the present invention may be prepared by saponifying and subjecting molecular distillation a deodorized soybean oil, as will be described in more detail below. The oil fraction so produced contains large amounts of phytosterols and tocopherols. Analysis of the unsaponifiable fraction of soybean oil by a color reaction test, a digitonide-formation reaction test, infrared and ultraviolet absorption spectra, and thin layer and gas liquid chromatography, reveals that the following substances are contained in the unsaponifiable fraction: plant sterols: β-sitosterol, stigmasterol and campesterol; natural tocopherols: α-, γ- and δ-tocopherols; fatty acids: lauric, myristic, palmitic and linoleic acids; and a small amount of squalane.

Various theories have been advanced to explain the physiological activity of α-tocopherol (vitamin E) which is a constituent of the unsaponifiable fraction of soybean oil, one of which is a biological antioxidant theory. Thus, it has been theorized that α-tocopherol has antioxidant properties because of the fact that animals deficient in vitamin E have in their body compounds which are considered to be peroxides of lipids. According to another theory, vitamin E directly takes part in an enzyme reaction, reducing cytochrome C in the presence of unsaturated fatty acids. Other interesting theories concerning vitamin E have been advanced, including a theory in which vitamin E is involved in the biosynthesis of and retention within body of ubiquinone. Another theory is that a lack of vitamin E gives rise to peroxidation of unsaturated aliphatic acids which are contained in living membranes, thus changing the transmittance or diffusing characteristics of the membranes.

As indicated above, the tocopherols are considered to have many unexpected physiological effects other than that of an antioxidant and may accordingly prove useful in a variety of applications as medicines. The medicinal action of the nonsaponifiable fraction of soybean oil used in the present invention appears to be due to the tocopherols which are present in large amounts in the nonsaponifiable fraction.

The unsaponifiable fraction of soybean oil of the present invention is a product which is obtained by the distillation and condensation of natural soybean oil. It is not now possible to artificially prepare an agent having the same composition as the distillate.

At the present time, it is also difficult to scientifically explain the reasons for the physiological potentiation of the components contained in the oil fraction or for the reduction of the undesirable side effects.

Experiments indicate that a nonsaponifiable fraction of soybean oil having a composition of about 45% by weight of plant sterols and about 20% by weight of tocopherols is preferred for use as an antilipemic agent. Thus, the nonsaponifiable fraction of soybean oil used in the present invention is prepared in a manner as to have about 45% by weight plant sterols and about 20% by weight tocopherols. The content of plant sterols and tocopherols depends upon the type of soybean oil used. The nonsaponifiable fraction of soybean oil of the present invention, can be prepared using crude fatty acids of soybean oil (deodorized soybean oil distillate) containing about 25% by weight of plant sterols and about 18% by weight of tocopherols by subjecting the same to esterification with methanol and then to molecular distillation to separate free fatty acids therefrom, then recovering the nonsaponifiable fraction which contains about 45% by weight of plant sterols and about 20% by weight of tocopherols.

The nonsaponifiable fraction of soybean oil used in the present invention is an opaque, brown semi-solid at room temperature and becomes a semi-transparent oily liquid when heated to a temperature higher than about 80°C. It also has a distinctive odor and tastes slightly sweet.

Although other pharmaceutically acceptable, orally administerable, carriers may be used, the nonsaponifiable soybean oil fraction used in the invention is preferably employed in an encapsulated granular form. In the granulation it is necessary to use as an absorbent a compound which is highly oil-absorptive and which does not adversely affect the stability of the tocopherols contained in the nonsaponifiable fraction. The use of silicates as carriers should be avoided since such carriers may cause liver troubles.

The preferred granules are those which easily disintegrate, or fall to pieces in water, and which can be readily filled into hard capsules by means of automatic filling apparatus.

A highly purified silicic acid anhydride which is obtained by thermal hydrolysis of silicon tetrachloride (available under the registered Trade Mark "Aerosil" No. 200—400) was used as the absorbent to make the antilipemic composition administered in the tests described below.

In a preferred procedure, the high purity silicic acid anhydride is first added to and mixed with the nonsaponifiable fraction of soybean oil, and the mixture is dried. The resultant product is then reduced to powdered form. An organic solvent such as chloroform, chlorothen, or methylene chloride, may be added to and kneaded with the powder which absorbs the solvent to produce granules having suitable properties for filling

into capsules by the use of an automatic capsule-filling machine.

In the above-described preparation of the granules, it is preferable to use together with the organic solvent an organic solvent-soluble binding agent such as polyvinylpyrrolidone, a copolymer of 2 - methyl - 5 - vinylpyridine-methacrylic acid, methylacrylate or the like, to improve the mechanical strength of the granules obtained and the quality of the tablets made therefrom. In order to further improve the solubility of the product in water, a small amount of a surface active agent such as sodium laurylsulfate, polyoxyethylene monostearate, polysorbate, or a derivative of castor oil or polyoxyethylene may be added to the mixture singly or in combination. Moreover, in order to stabilize the tocopherols in the nonsaponifiable fraction, a small amount of antioxidants and synergists of the antioxidants such as vitamin C, citric acid, etc. may be added.

A small amount of lubricant such as magnesium stearate may also be added to the granular product to facilitate filling into capsules by means of conventional automatic capsule-filling machines.

Granules produced by this technique usually contain about 50% by weight of the nonsaponifiable fraction of soybean oil. They are readily disintegrated by water, and they offer the further advantage that the stability of the tocopherols contained therein is excellent.

The invention is illustrated by the Examples and Tests which follow.

Example 1.

Preparation of the Nonsaponifiable Fraction of Soybean Oil:

A coarse fatty acid fraction (soybean oil-deodorized distillate) of soybean oil, containing about 25% by weight of plant sterols and about 18% by weight of tocopherols, was reacted with methanol in the presence of concentrated sulfuric acid at a temperature of 68°C for 3 to 4 hours to form an ester product. The excess methanol was removed from the reaction product, which was then washed with water at a temperature of 170° to 180°C, and condensed and purified by molecular distillation at a temperature of 170° to 180°C under a vacuum of 20 to 50 mmHg. The purified nonsaponifiable fraction of soybean oil was then analyzed by a color reaction test, a digitonin precipitation method, infrared and ultraviolet absorption spectra and a thin layer and gas chromatography. The test results are shown as follows:

Plant sterols including campesterol, stigmasterol and β-sitosterol . . . about 45% by weight

Tocopherols containing α-, γ- and δ-tocopherol . . . about 20% by weight

Squalene . . . slight amount

The tocopherols in the nonsaponifiable fraction were identified as natural tocopherols, on the basis of the data given in Table 1 below.

TABLE 1

		α-tocopherol	γ-tocopherol	δ-tocopherol
Color reaction	Emmerie-Engel's reaction		(Red color)	
	Furter-Meyer's reaction		(Reddish brown color)	
Thin layer* chromatography Test	(Solvent) Chloroform	Rf 0.65 – 0.7	Rf 0.45 – 0.5	Rf 0.25
	(Solvent) n-hexene ether.glacialacetic acid	Rf 0.31	Rf 0.25	Rf 0.18
Ultraviolet absorption spectrum (Maximum absorption Wavelength)	Soy-sterol	295 nm	296 – 297 nm	297 – 298 nm
	Control	290	296 – 297	296 – 297
	Merck Index 8th	294	298	298

*. . . . With chloroform, weak spots at Rf 0.98 and 0.92 were further found and these were confirmed as compounds similar to tocopherols having a phenolic OH group.

The fatty acid esters contained in the non-saponifiable matter were identified from the test results of gas liquid chromatography as including esters of lauric acid, myristic acid, palmitic acid, linoleic acid and the like. The test results are shown in Table 2 below:

TABLE 2

Peak No.	Retention time (min)	Assignment	Peak No.	Retention time (min)	Assignment
PA_1	50	Lauric acid	PA_4	280	Stearic acid
PA_2	8.9	Myristic acid	PA_5	31.4	Oleic acid
PA_3	15.7	Palmitic acid	PA_6	39.0	Linolic acid

The nonsaponifiable fraction of soybean oil has a brownish color and is an opaque semi-solid at room temperature. It has a distinctive odor and tastes slightly sweet. When the fraction is heated to a temperature higher than about 80°C, it turns into a translucent oily liquid. The nonsaponifiable fraction was subjected to a number of tests, as follows:

Test 1—Solubility.

The nonsaponifiable fraction shows selective solubility in various solvents. It is extremely soluble in chloroform, easily soluble in ether, sparingly soluble in acetone, hardly soluble in ethanol and almost insoluble in water. The solubility data in these solvents is as follows:

Solvent	*Solubility*
Chloroform	1 g/0.9 ml
Ether	1 g/2.0 ml
Acetone	1 g/500 ml
Ethanol	1 g/5000 ml
Water	1 g is insoluble in 10,000 l of water

Test 2—Temperature Stability.

Temperature, humidity and light stabilities of the nonsaponifiable fraction have been determined. With regard to temperature stability, where the unsaponifiable fraction of soybean oil was placed in a transparent wide-mouthed medicine bottle and the bottle sealed with a metal stopcock and allowed to stand at room temperature over a two year period, the nonsaponifiable fraction changed color from brown to yellowish brown but showed no shift in the maximum and minimum ultraviolet absorption spectra, at 295 nm and 261 nm respectively in ethanol solution. Furthermore, the content of tocopherols was found to be reduced only by about 2% by weight after the first year and by about 10% after two years. Where an excipient such as SiO_2 was granulated and encapsulated, no changes in color or in tocopherol content were noted over a like period of time.

Test 3—Humidity Stability.

In order to test the humidity stability of the non-unsaponifiable fraction, the nonsaponifiable fraction was granulated and encapsulated by the procedure of Example 2 below, and the resultant granules were placed in nine different containers, the atmosphere within each container being regulated using different saturated salt solutions. After exposure within the containers for 15 days, the hygroscopicity of the respective granules was measured.

The results of the humidity tests are given in Table 3 below.

TABLE 3

		Relative Humidity during Reservation (R.H.) %								
	Control	20.4	40.2	53.7	70.2	79.1	82.3	85.6	90.3	95.5
Hygroscopicity	Nil	Nil	Nil	Nil	Nil	Yes	Yes	Yes	Yes	Yes
Color	Pale Yellow	Pale Yellow	Pale Yellow	Pale Yellow	Yellow	Yellow	Yellow	Yellow	Dark Yellow	Dark Yellow

The saturated salt solutions employed in the above measurements were as follows:

Salt	*Relative Humidity (%)*
CH_3COOK	20.4
CrO_3	40.2
$NaBr\text{-}2H_2O$	53.7
$NaNO_3$	70.2
$(NH_4)_2SO_4$	79.1
KCL	82.3
K_2CrO_4	85.6
KNO_3	90.3
K_2SO_4	95.5

The saturated salt solutions shown in the left-hand column above, maintain the respective Relative Humidities shown in the right-hand column when kept in sealed bottles at 37°C.

As is apparent from Table 3, the encapsulated nonsaponifiable fraction of soybean oil is stable below a relative humidity of about 60%. The data indicates that the critical humidity for stability of the nonsaponifiable fraction of soybean oil is 63.1%.

Test 4—Light Stability.

The light stability of the nonsaponifiable fraction was measured, with and without encapsulation, by irradiating samples by means of a low temperature "Xenon" (registered Trade Mark) fade meter with a light quantity (about 180 Langley, ultraviolet ray having 300—400 nm) corresponding to that of 10 day's direct sunlight, averaged through a year. During the irradiation period, the samples were observed for changes in color and odor, and were subjected to qualitative analysis, to measurement for ultraviolet spectra absorption, to thin layer chromatography and to quantitative analysis. In the case of those samples of the nonsaponifiable fraction of soybean oil not encapsulated, the color changed from brown to yellowish brown, and the wavelengths of the maximum and minimum absorption spectra at 295 nm and 261 nm in ethanol solution, respectively, did not shift at all, though the ratio of the light absorption spectra increased by about 3.5% after 5 days' irradiation and by about 8.7% after 10 days' irradiation. The content of tocopherols was reduced in an amount of about 6% after 5 days' irradiation, and of about 10% after 10 days' irradiation. On the other hand, with the encapsulated samples, prepared as in Example 2 below, the degree of change was smaller as compared with that of the samples not encapsulated: the ratio of ultraviolet absorption spectra was increased by 3.6% after irradiation for 5 days and by 5% after irradiation for 10 days; and the tocopherol content was reduced by 2% after irradiation for 5 days and by 6% after irradiation for 10 days.

TOXICITY TESTS

It will be appreciated from the above test results that antilipemic compositions containing therapeutically effective amounts of the unsaponifiable fraction of soybean oil should be kept in a dark storage place. Experiments show that when the nonsaponifiable fraction of soybean oil (both encapsulated and non-encapsulated) is placed in a sealed container and shielded from light, it retains its therapeutic effectiveness for longer than two years.

The toxicity of the nonsaponifiable fraction of Example 1 was examined by means of an acute toxicity test, a subacute toxicity test and a chronic toxicity test, which tests were conducted in the manner described below. The antilipemic compositions employed were prepared as described in Example 2.

Test 5—Acute Toxicity Test

A number of dd mice (female and male) having a weight of 12 to 18 g and Wistar rats (female and male) having a weight of 60 to 90 g, both being procured when 4 weeks old and bred in a laboratory for 1 week, were employed as the test animals. Five mice and five rats were taken as each group in the test. A sesame oil solution of the nonsaponifiable fraction of soybean oil was dosed by stomach tube to these animal groups in accordance with the following prescriptions and each animal group thus treated was kept in a breeding box maintained at a temperature of 22 ± 2C with a relative humidity of 55±5%.

Mouse: 8.0 g/kg through mouth
4.0 g/kg through hypodermic injection
2.0 g/kg through abdominal injection

Rat: 8.0 g/kg through mouth
2.0 g/kg through hypodermic injection
1.0 g/kg through abdominal injection

72 hours after dosing, the life signs of the test animals were observed and for 10 days thereafter to determine the LD_{50} value. The test results are as follows:

LD_{50} (median lethal dosage): 72 hours after dosing, all of the mice and rats were alive and even after the subsequent 10 days, none had died. Thus, it was impossible to calculate a LD_{50} value.

Toxic symptoms: No differences between the test animals and normal animals were observed with regard to toxic symptoms or behavior.

Test 6—Subacute Toxicity Test.

A number of Wistar male rats (weight: 120—160 g) and female rats (weight: 105—135 g), purchased when four weeks old and

bred in a laboratory for 1 week, were employed as test animals. Ten male and ten female rats were taken for each group in the test. The unsaponifiable fraction of soybean oil was mixed with a powdered foodstuff and the mixture was dosed to each of the groups in the different amounts shown below. The dosed rats were kept in breeding boxes maintained at a temperature of 22±2°C with a relative humidity of 55±5%. The breeding boxes were ventilated 10 times per hour.

Daily Dosing Amounts: 9000 mg/kg
4500 mg/kg
2250 mg/kg

The above differing dosing amounts were determined on the basis of results of preliminary experiments wherein rats were dosed with the unsaponifiable fraction over 2 weeks. The antilipemic composition of the present invention was administered by mouth to each group of rats (males and females being kept separately) daily in the three different dosing amounts mentioned above, by mixing the composition with a powdered foodstuff produced by Nippon Clare K.K. The dosing test was continued for 1 month, periodically measuring the weight of each test animal. The amounts of feed and water taken by each test animal were also monitored and the animals were observed for toxic symptoms. At the end of the two week dosing period, the test animals were subjected to a urinalysis (for determining pH, protein and sugar values), a blood test (for determining number of red blood corpuscles, number of white blood corpuscles, amount of hemoglobin, and white blood pattern), a patho-morphological study (for examination of main organs by dissection and measurement of weight), and a biochemical study of serum (for measurement of GOT, GPT, Al-P, Ch-E, T.Ch, F.Ch, Na^+, K^+, Cl^-, serum protein and blood sugar).

The test results are summarized as follows.

1) General symptoms were the same as those of the controls with no deaths or toxic symptoms.

2) No significant changes in weight, or in the amount of feed and water consumed was noted for any animal.

3) No significant differences between the test groups and the control group were discovered by the blood tests.

4) The biochemical study of the blood serum of each test animal revealed no differences that could be attributed to differences in the amounts of the nonsaponifiable fraction administered.

5) No adverse effects on the weight of the organs or in the patho-morphological study were noted.

Test 7—Chronic Toxicity Test.

A number of Donryu male rats (weight 90—130 g), purchased when four weeks old and raised for a suitable period of time, were used as test animals. Ten rats were taken for each group. The nonsaponifiable fraction of soybean oil was mixed with a powdered foodstuff, and the mixture was fed to each of the test animals in the different amounts shown below. The rats thus fed were kept in breeding boxes maintained at a temperature of 22±2°C with a relative humidity of 55±5%.

Daily Dosing Amount: 9,000 mg/kg
6,000 mg/kg
3,000 mg/kg

In one group (of ten rats), three rats were dosed for 13 weeks and seven rats for 27 weeks. During the test priod, the weight and feed intake of each rat were monitored while observing for toxic symptoms. After completion of the respective test periods, the test rats were subjected to a urinalysis (pH, protein, sugar), a blood test (number of red blood corpuscles, number of white blood corpuscles, amount of hemoglobin, white blood pattern), a patho-morphological study (examination of main organs by dissection and measurement of weight) and a biochemical study of the serum (measurement of GOT, GPT, Al-P, Ch-E, LDH, T.Ch, F.Ch, Na^+, K^+, Cl^-, serum protein and blood sugar). The test results are summarized below.

1) General symptoms of the rats fed the antilipemic composition were the same as those of the controls, with no deaths or toxic symptoms.

2) No optical-microscopic changes in the artery systems coronary artery, renal artery, etc., were noted.

3) In the kidneys no marked differences were observed in fat deposition, in the amount of glycogen, in generation of stellate cells, or in the parenthemal cells as compared to the control group.

4) No changes in myelopoietic functions were noted.

5) The biochemical examination revealed no unusual changes.

6) No significant differences were observed with respect to weight, feed and water consumption, the items of the blood test, or with respect to the weight of the organs examined.

It will be appreciated from the above-summarized test results that the antilipemic agent of the present invention is extremely nontoxic, so that it is possible to administer the agent over a long period of time without incurring adverse effects.

The medicinal effects of the antilipemic

composition of the present invention have been determined by laboratory tests on both animals and clinical cases. Several of such tests are described below.

Test 8—Pharmacological Effects.

Testing method: A number of male rabbits were employed as test animals. The rabbits were purchased at a weight of about 2.0 kg and raised for about 2 weeks, allowing them to become acclimatized to their new environment before being employed as test animals. Thirteen rabbits were employed in the test and the rabbits were divided into a control group and a test group. A solid foodstuff (produced by Nippon Clare K.K.), containing 1% by weight of cholesterol, was fed each day to both groups of rabbits in the amount of 100 grams per rabbit. Water was supplied *ad libitum* by means of an automatic water-feeding apparatus. The antilipemic composition was administered to the test group in capsule form in the amount of 1.7 g per day for 12 weeks; the control group was fed potato starch in the same manner. The level of lipids in the blood serum of all the animals was measured in the manner described below using blood sampled from the ear vein, prior to the start of the test period and, thereafter, every two weeks during the test period.

Total Cholesterols	:	Kitamura's modified Zak-Henry method
Free Cholesterol	:	Digitonin Method
Neutral Fats	:	Van Handel-Kawade's improved Yamamoto Method
Phospholipids	:	Nakamura's modified Allen Method
Lipoprotein	:	Electrophoresis Method

A) Change in weight

The weights of the composition-dosed group rabbits as well as control group rabbits increased normally. In this respect, no differences were noted between the two groups.

B) Lipids in serum

1) Cholesterol

A remarkable increase in the amount of total cholesterol and free cholesterol was noted in the control group, while the group to which the antilipemic composition was administered showed a relatively small increase in both total and free cholesterol which was about half that of the control group. Fig. 1 of the accompanying drawings is a graphical representation of changes in the amount of total cholesterol in serum versus time elapsed during the test period for both the agent treated group and the control group. Fig. 2 of the accompanying drawings is a graphical representation, similar to Fig. 1, of changes in the amount of free cholesterol in serum versus time. In these figures, "ST—2" designates the antilipemic composition of the present invention containing a nonsaponifiable fraction of soybean oil. The data shown in Figs. 1 and 2 indicates that the antilipemic composition of the present invention effectively suppresses the increase of cholesterol in serum.

2) Phospholipids:

Fig. 3 of the accompanying drawings is a graphical representation of changes in amount of phospholipids in the serum versus time elapsed, in the test period for both the composition treated group and the control group. As is apparent from Fig. 3, the antilipemic composition of the present invention is also effective in suppressing the increase of phospholipids in serum.

3) Neutral Fat:

Fig. 4 of the accompanying drawings is a graphical representation of changes in the amount of neutral fat in the serum versus time elapsed, for both the composition-treated group and the control group. As shown in Fig. 4, the neutral fat value for the composition treated group was slightly higher than that of the control group before commencement of the test, but became lower than that of the control group after 6 weeks of the dosing. After 12 weeks, the neutral fat value of the composition-dosed group was reduced to half that for the control group.

4) Lipoprotein in Serum:

Fig. 5 of the accompanying drawings is a graphical representation of changes in the amount of lipoproteins in the serum versus time elapsed for the agent-treated group and for the control group. As shown in Fig. 5, in the control group, the ratio of β-lipoprotein to α-lipoprotein sharply increased, while in the composition-dosed group, the ratio increased only slightly and reached a constant value after 8 weeks of the dosing. Thus, the data indicates that the antilipemic composition of the present invention also suppresses the increase of formation of lipoprotein in serum.

C) Lipid *in viscera* (liver cholesterol)

As shown in Table 4, below, the total amount of cholesterol *in viscera* and free cholesterol *in viscera* of the composition-treated group were reduced to low values, differing significantly from the control group.

TABLE 4

Group	Liver Cholesterol		
	Total (mg/g)	Free (mg/g)	Free %
Control	4.57 (± 0.890)	1.09 (± 0.166)	24.84 (± 1.433)
ST–2*	1.39** (± 0.343)	0.47** (± 0.068)	38.24** (± 2.694)

* – The antilipemic composition of the present invention

** – There exists a significant difference in p 0.01 ± S.E. (S.E. = standard error)

D) *Plant sterols contained in serum and liver*

The amount of total cholesterol contained in the serum and liver was measured by a colorimetric analysis in which a compound having a sterol ring induces a reaction. If plant sterols are present in a sample, a color reaction identical to that for cholesterol takes place. Therefore, where the amount of total cholesterol in the serum was determined after administration of the antilipemic composition of the present invention, this total cholesterol value is regarded as a sum of values for cholesterol and plant sterols. Accordingly, the use of a value for total cholesterol does not reflect a correct diagnosis for lipemia. Thus, in order to correctly determine the cholesterol level in each viscus and serum sample, it is necessary to correctly determine and subtract the amount of plant sterols. The plant sterol value can be determined using an FID gas liquid chromatography in combination with thin layer chromatography.

Test 9A—Tests on Laboratory Animals

Four groups of white rats were used in this test. One control group was fed with a cholesterol-free foodstuff while a second control group was fed a 0.5% cholesterol-containing foodstuff. A third group was fed with a cholesterol-added foodstuff, containing 1.5% by weight of plant sterols, and the fourth group was fed with a cholesterol-added foodstuff containing the antilipemic composition of the present invention. These foodstuffs were fed to the respective groups for four weeks. At the conclusion of the four week test period, the cholesterol values in the serum and as liver lipids were determined, and the lipid fractions of the serum and liver samples were analyzed by FID gas-liquid chromatography in combination with thin layer chromatography. The test results are shown in Table 5, below. It is apparent from Table 5 that about 50% by weight of the total cholesterol value represented substituted plant sterols. Accordingly, the value which is obtained by subtracting the plant sterol value from the total cholesterol value is considered to be a true cholesterol value for serum and as liver lipids. The data shows that when the antilipemic agent of the present invention is fed to rats, the true cholesterol value is reduced to a remarkable degree.

TABLE 5

Components and level of sterols in serum (plasma) and liver lipids of rats fed with soy-sterols

(Test 9A)

	Group	(Plasma) Serum Sterol mg/dl				Liver Sterol mg/g			
		Total Chole-sterol	Chole-sterol	Stigma-sterol	β-sito-sterol	Total Chole-sterol	Chole-sterol	Stigma-sterol	β-sito-sterol
I	Food stuff without cholesterols	160	160	–	–	2.8	2.8	–	–
II	Food stuff containing 0.5% by weight of cholesterols	195	195	–	–	60.0	60.0	–	–
III	Food stuff containing 1.5% by weight of fractions mainly composed of plant sterols	160	87	73		6.4	4.6	1.3	0.5
IV	Feed stuff containing 1.5% by weight of antilipemic agent of the invention	147	81	50	16	5.6	1.7	2.8	1.1

Test 9B—Clinical Tests.

The antilipemic composition used in tests 9(A) and 9(B) and in the clinical test (test 10) described hereinafter, was administered orally in capsule form, each capsule containing 200 mg of the unsaponifiable fraction of soybean oil.

A) Test on Healthy Subjects

A predetermined amount of food (about 2700 Cal per day), including meats of high fat content (fat: 40%), was fed to each of nine healthy subjects for 6 weeks. During this test, a placebo was given to the subjects daily for the first one week, and during the next four weeks, the antilipemic composition was administered. During the last week, the placebo was again administered. In each case, 6 capsules were used daily for dosing. The values for total cholesterol and for plant sterols in the serum of the tested subjects were determined by Zak-Henry's method and by the method previously described in Test 6A respectively.

It was determined that plant sterols

accounted for 10% by weight of the total cholesterol in the serum. Furthermore, one week after completion of the treatment with the antilipemic composition of the present invention, plant sterols could not be detected in the serum in any amount. The test results are shown in Table 6 below.

TABLE 6

Changes in biochemical components of serum lipids caused by Placebo and ST–2

Items examined	Dosed amount	Value before dosing	Placebo		**** ST – 2		Placebo
			Value after one week	Value after one week	Value after two weeks	Value after three weeks	Value after one week
GOT	(u)	19	12	9	12	9.6	13.4
GPT	(u)	15	11	6	9	4.2	11.3
ALP	(u)	7.1	5.0	4.8	4.6	5.7	4.5
LDH	(u)	280	200	250	280	260	290
TCH	(mg/dl)	197±25	228±36	240±40	240±40	240±29	240±62
PS*	(mg/dl)	0	0	21	20	24	0
β-Lip. pro.	(mg/dl)	325±122	321±98	300±82	230±94	246±106 O	280±90
T.T.**	(γ/ml)	6.1±4.7	7.5±1.0 Θ	4.8±3.0	9.2±3.1	15.3±4.3 Θ	17.1±4.6
TBA***	(OD/ml)	0.058	0.05	0.072	0.068	0.06	0.06

* PS: Phytosterol

** T.T. : Total tocopherol

*** TBA: Thiobarbituric acid value

**** ST – 2 : This drug

O : $p < 0.05$

Θ : $p < 0.01$ where p represents confidence limits.

u : International unit

B) Test on Patients

This test was conducted with 45 patients who were hypertensive and had total serum lipid cholesterol levels higher than 220 mg/dl. Nine capsules of a placebo were administered orally daily to each patient for the first three weeks of a six week test period, and nine capsules of the antilipemic composition used in the previous tests were orally administered daily for the next three weeks. The total cholesterol in the form of blood serum lipids was determined by ZakHenry's method, and the lipid fractions of the same serum were analyzed by a FID gas liquid chromatograph used in combination with thin layer chromatography. The results are shown in Table 5.

As is apparent from Table 5, the total serum lipid cholesterol value was reduced by 13.5% on average ($P<0.05$) by treatment with the antilipemic composition of the present invention, as compared to the placebo control. Furthermore, if the substituted plant sterols, about 9% on average, are subtracted from the total cholesterol value, the true serum lipid cholesterol value becomes lower by 22.5% than the corresponding value for the placebo control. While the placebo was being administered, no plant sterols were detected in the serum.

5) Pathological Observation

a) Visceral Observation

Heart: Fat deposition was noted in portions of the cardiac apexes and coronaries of both groups, but the number of cases showing such deposits was smaller for the antilipemic composition-treated group.

Spleen: A milk-white substance was found in both groups, but in a greater amount in the control group.

TABLE 7

Case No.	Name	Sex	Age	Sterols: Before dosing	Sterols: After dosing of Placebo for three weeks mg/dl	Sterols: After dosing of ST–2 for three weeks mg/dl	Sterols: Amount reduced mg/dl	Plant sterols (%): P*	Plant sterols (%): ST–2****	Total Tocopherols mg/dl	Liver Functions: GOT	Liver Functions: GPT	Liver Functions: ALP	Liver Functions: LDH
1	T.T.	M**	36	225	220	218	−2	0	5	0.5	32	11	8.6	
2	S.T.	M	42	240	245	200	−45	0	3	0.35	16	8	5.6	280
3	K.K.	M	50	232	270	240	−30	0	20	1.35				
4	T.T.	M	48	238	235	215	−20	0	4	0.45				
5	M.M.	M	59	293	285	230	−55	0	10	0.70	15	8	5.8	
6	T.T.	F***	56	307	310	300	−10	0	25	0.45	74	29	12	560
7	S.O.	F	45	294	290	220	−70	0	4	0.85	26	44	4	310
8	N.T.	F	40	271	275	240	−35	0	8	1.90	13	7	3	200
9	I.Y.	F	52	255	265	250	−15	0	21	1.70	26	29	4.7	130
10	N.Y.	M	74	290	280	230	−50	0	18	1.70	13	8	4.9	210
11	H.R.	F	66	277	270	220	−50	0	15	1.70	16	4		
12	K.T.	M	62	198	205	200	−5	0	10	1.20				
13	W.T.	M	19	275	270	250	−20	0	8	1.60	60	13	4.6	300
14	K.T.	F	78	285	290	240	−50	0	6	1.50				

TABLE 7 (Continued)

Case No.	Name	Sex	Age	Sterols: Before dosing	After dosing of Placebo for three weeks mg/dl	After dosing of ST–2 for three weeks mg/dl	Amount reduced mg/dl	Plant sterols (%): P*	Plant sterols (%): ST–2****	Total Tocopherols mg/dl	Liver Functions: GOT	GPT	ALP	LDH
15	A.K.	M	34	235	250	225	−25	0	0	0.70	10	5	4.5	200
16	I.K.	M	58	275	260	210	−50	0	7	0.90	64	58	6.7	240
17	O.S.	M	65	283	275	198	−77	0	5	1.20	30	22	3.5	280
18	S.K.	F	45	260	275	230	−45	0	3	1.30	25	22	9.9	240
19	T.S.	M	22	260	268	262	−6	0	20	1.10	38	15	13.3	400
20	M.S.	F	43	235	255	231	−24	0	22	0.80	30	18	5.3	430
21	G.E.	F	42	259	257	218	−39	0	15	2.05			3.2	280
22	H.S.	M	53	290	288	235	−33	0	28	1.55	126	112	13.3	280
23	K.O.	M	59	292	296	270	−26	0	25	1.20	13	9	3.8	220
24	M.K.	M	72	302	312	260	−52	0	22	0.90	27	20	6.7	430
25	R.H.	M	66	315	305	235	−70	0	0	1.70	15	8	5.2	210
26	S.K.	F	69	205	208	208	0	0	4	0.60	18	11	4.3	700
27	R.T.	F	66	305	300	310	+10	0	0	0.70	28	38	12.5	130
28	K.K.	M	73	289	297	227	−50	0	22	0.75	60	15	11.0	490

TABLE 7 (Continued)

Case No.	Name	Sex	Age	Sterols: Before dosing	Sterols: After dosing of Placebo for three weeks mg/dl	Sterols: After dosing of ST–2 for three weeks mg/dl	Sterols: Amount reduced mg/dl	Plant sterols (%): P*	Plant sterols (%): ST–2****	Total Tocopherols mg/dl	Liver Functions: GOT	Liver Functions: GPT	Liver Functions: ALP	Liver Functions: LDH
29	U.S.	F	60	270	272	251	−21	0	21	0.65	32	22	4.3	350
30	T.M.	M	67	260	268	240	−28	0	8	1.10	36	16	5.6	320
31	T S.	M	46	265	255	195	−60	0	7	1.35	28	28	7.8	150
32	A.A.	F	63	269	265	196	−69	0	0	0.95	70	19	11.0	400
33	G.O.	M	49	240	245	187	−58	0	5	1.25				
34	T.I.	M	66	276	280	220	−60	0	0	1.60	66	28	12.2	270
35	I.K.	M	63	240	235	238	+3	0	8	1.15	33	52	4.3	290
36	I.I.	F	58	207	215	200	−15	0	7	0.80	10	15	3.8	200
37	H.A.	F	62	220	228	230	+2	0	6	1.90	18	16	2.9	280
38	S.T.	M	60	223	219	200	−19	0	2	1.70	35	15	5.3	240
39	T.F.	M	71	300	305	220	−85	0	7	0.75	26	22	7.2	230
40	S.S.	M	61	305	309	300	−9	0	8	0.95				
41	K.Y.	F	55	325	320	295	−25	0	0	1.35	57	48	3.6	450
42	N.T.	F	53	290	300	240	−60	0	0	1.15	26	20	6.7	210

TABLE 7 (Continued)

Case No.	Name	Sex	Age	Sterols: Before dosing	Sterols: After dosing of Placebo for three weeks mg/dl	Sterols: After dosing of ST–2 for three weeks mg/dl	Sterols: Amount reduced mg/dl	Plant sterols (%): P*	Plant sterols (%): ST–2****	Total Tocophenols mg/dl	Liver Functions: GOT	GPT	ALP	LDH
43	M.K.	M	69	229	225	190	−35	0	0	1.55	14	6	3.1	290
44	S.K.	F	57	250	260	190	−70	0	0	1.25	35	22	5.6	390
45	M.M.	F	61	266	270	195	−75	0	0	0.80	38	13	6.7	300
Average value ± Standard				265	267	231	−36	0	9.1					
Deviation				±30	±30	±31			±8.5					
P					P>0.05	P<0.05	Reduced ratio 13.5%							
Total serum lipid cholesterols														

Note.
*: Placebo **M: Male ***F: Female ****ST–2: This drug

Liver: Almost all of the control group were found to have a fatty liver, while most of the antilipemic agent treated group showed a normal liver color.

Aorta: In the control group, the degree of arteriosclerosis was greatest at the arch region with a downwardly weakening tendency toward the thorax and the abdominal region. Distinct fat deposits were also found in cardiac artery arteriomesenterial and renal artery openings. On the other hand, in the antilipemic composition treated group, these symptoms were found in only 1 or 2 cases.

b) Weight changes of viscera

With regard to the kidney, lung and heart, no differences between the two groups was noted, but with regard to the liver, adrenal glands and spleen, the weights of these organs for the antilipemic composition-treated group was slightly smaller than that of the control group. A significant difference was noted only in liver weight.

c) Histological Examination

The aorta (arch region), heart, lung, spleen, and adrenal glands were histologically examined.

Aorta: In the control group, generation or degeneration of foam[2/] cells took place. Furthermore, fat was diffusively deposited on portions of the median membrane in droplet or oval form. Additionally, a few instances of atheromatous degeneration within the aorta were noted which showed swelling and breakage of elastic fibers, and the proliferation or growth of glue-like fibers. In some cases within the control group, foam cells co-existed with a relatively large complex of fat at the uppermost layer of the inner membrane and with fine droplets on the lower layer, indicating a slight degree of atheroma. In the other cases, there was either almost no change or a slight infiltration of lipids in the endothelia of the inner membrane.

Within the antilipemic composition-treated group, in only one severe case were granular fat deposits noted on endothelium of the aorta. Foam cells were also noted in the singular case. In the other cases within the treated group, no such conditions were noted.

Lung: Oedemalosous hypertrophy on the inner membrane of the artery walls was noted in two cases within the control group. No such symptoms were noted in any case within the treated group.

Heart: No fat deposits were noted on the myocardial fibers in any case, except for a slight accumulation of fat on the epicardium in a few cases within the control group. Membranic edemas on the aortic arch were noted in 4 cases within the control group, in which cases athermatous symptoms were distinctly visible. In one case within the composition-treated group an intimal edema was noted, slightly dyed with eosin.

Spleen: Nest-like fat deposits under the membrane were noted in four cases in the control group and in three cases in the composition-treated group, although the degree was different. In those three composition-treated cases, there was a slight hypertrophy of the spleen.

Liver: Although highly fatty liver symptoms were observed with the naked eye, there actually were only small deposits of fat globules in the liver cells and a pattern of circumferential pimelosis was observed. The fat deposits were noted mainly in the stroma or interstitial cells and in Kupffer's stellate cells, particularly in and around the center of the vein of the Glisson's capsule These symptoms of the liver were, in a larger degree, found in the control group, and generally only slightly in the composition-treated group.

Test 10—Clinical Tests.

The following clinical tests were conducted at the Tokyo Medical College.

Test Method: 38 patients having diverse symptoms such as an ischemic heart disease, hypertension, diabetes, acute hepatitis, and gastric ulcers were subjected to the clinical tests. The total cholesterol value in the serum was within the range of 205 to 335 mg/dl before treatment and had an average value of 260.4 mg/dl. 26 patients showed a total cholesterol level higher than 250 mg/dl, and 12 patients a value within a range of 205 to 248 mg/dl. Six capsules of the antilipemic composition prepared as described in Example 2 were fed orally to each of the patients daily for 4 to 20 weeks, or for 7.6 weeks on the average. 24 patients were administered an inactive placebo, instead of the agent, during the period between the 8th and 12th weeks, inclusive.

Results:

Serum Cholesterol Value:

The average value for serum cholesterols before treatment with the composition of the present invention was 260 mg/dl, which value was reduced to 225.9 mg/dl two weeks after commencement of the treatment and to 229.9 mg/dl four weeks after. The value rebounded to slightly higher levels, i.e., 232.2 mg/dl and 240 mg/dl respectively, 6 weeks and 8 weeks after the commencement of the

[2/] A foam cell is defined by Webster's as "A swollen vacuolated reticuloendothelial cell filled with lipide inclusions and characteristic of certain conditions involving disturbance of lipide metabolism.

treatment. In the 12th week after the placebo had been administered in place of the antilipemic composition for 4 weeks, the value rose to 244.8 mg/dl.

β-lipoprotein:

i) Fried Hoeflmayr's Method

The average value for β-lipoprotein before treatment was 592.3 mg/dl, 2 weeks after commencement of the treatment it fell to 527.1 mg/dl, 4 weeks after 529.8 mg/dl, 6 weeks after 535.1 mg/dl and 8 weeks after 550 mg/dl. Thus, the value was reduced during the 2 to 6 week interval after commencement of the treatment. In the cases where the placebo was used after the eighth week, the values were 524.8 mg/dl 10 weeks later, and 520.7 mg/dl 12 weeks later. Thus, there appeared to be no rebound phenomenon.

ii) Capillary Precipitation Method

The average value for 11 test cases was 4.5 mm before commencing treatment with the composition of the present invention and was reduced to 3.7 mm 4 weeks after commencement, and to 4.0 mm 8 weeks after. Furthermore, in five cases, where the composition was administered for 4 weeks and then substituted with a placebo, the average value was 3.7 mm 8 weeks after. Therefore, no rebound phenomenon was evident.

GOT, GPT:

When GOT and GPT were studied in 38 cases, the average value of GOT before treatment was 31.2 and 25.1 after treatment, and those of GPT, before and after the treatment, were 32.9 and 26.2, respectively.

The Meulengracht's value and the ALP value were studied in 38 cases. The average of the Meulengracht's values was 6.9 before treatment with the present composition and 6.8 after the treatment, and average values of ALP before and after the treatment were 8.8 and 8.6, respectively. These values remained relatively constant before and after the treatment, thus indicating that the composition of the present invention did not produce any ill effects.

Secondary Action:

No secondary effects were observed

An example illustrating the preparation of a granular composition in accordance with the invention will now be given.

Example 2.

470 g of the nonsaponifiable fraction of soybean oil prepared in Example 1, 20 g of vitamin C, 10 g of citric acid, 40 g of calcium cellulose glycolate, 20 g of sodium laurylsulfate, 10 g of polyoxyethylene monostearate and 600 ml of a halogenated hydrocarbon solvent were measured and sufficiently mixed to form a suspension. 390 g of "Aerosil" No. 200—400 ("Aerosil" is a registered Trade Mark) were added and mixed with the suspension while agitating. The mixture was then dried at a temperature of about 50 to 60°C to give a solid material. The solid product was then pulverized to reduce to powdered form. To the powder was added 600 ml of a chlorothen-ethanol solution containing 40 g of polyvinylpyrrolidone. The resultant mixture was kneaded and then granulated using an ECK pelleter. The resultant granules were then dried at about 50°C to give a non-tacky product. The content of the nonsaponifiable fraction of soybean oil in the granular product was 47% by weight. The granules readily disintegrated in water. Additionally, the stability of the tocopherols in the granular product was excellent.

WHAT WE CLAIM IS:—

1. An antilipemic composition comprising a nonsaponifiable fraction of soybean oil and an orally administerable carrier, said nonsaponifiable fraction containing about 45% by weight of plant sterols, including campesterol, stigmasterol and β-sistosterol, and about 20% by weight of tocopherols.

2. A composition according to Claim 1, wherein said carrier comprises a silicic acid anhydride.

3. An antilipemic composition, according to Claim 1, and substantially as hereinbefore described.

TREGEAR, THIEMANN & BLEACH,
Chartered Patent Agents,
Enterprise House,
Isambard Brunel Road,
Portsmouth PO1 2AN,
— and —
49/51, Bedford Row, London WC1V 6RU,
Agents for the Applicants.

Printed for Her Majesty's Stationery Office by the Courier Press, Leamington Spa, 1976.
Published by the Patent Office, 25 Southampton Buildings, London, WC2A 1AY, from which copies may be obtained.

1427253 COMPLETE SPECIFICATION
2 SHEETS *This drawing is a reproduction of the Original on a reduced scale*
Sheet 1

FIG. 1

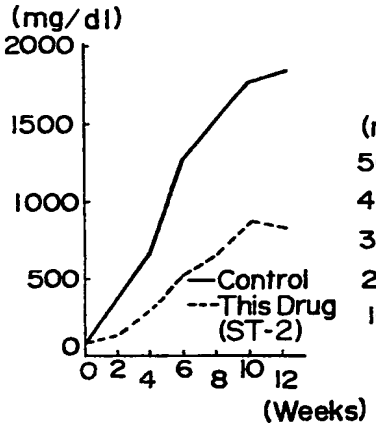


FIG. 2

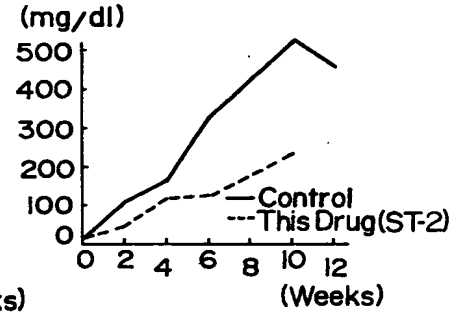


FIG. 3

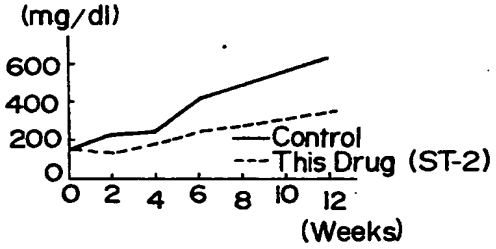


FIG. 4

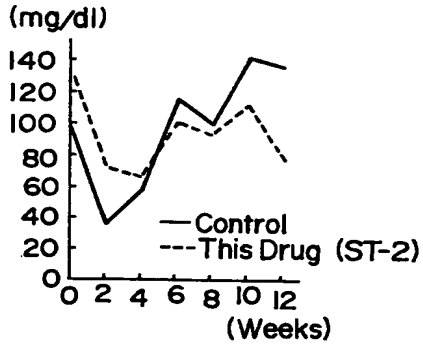
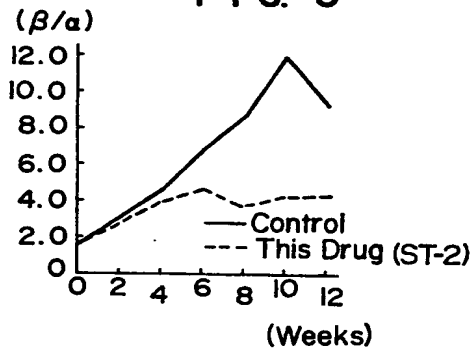

(mg/dl)
140
120
100
80
60
40
20
0
0 2 4 6 8 10 12
(Weeks)
—Control
---This Drug (ST-2)

FIG. 5


(β/α)
12.0
10.0
8.0
6.0
4.0
2.0
0
0 2 4 6 8 10 12
(Weeks)
—Control
--- This Drug (ST-2)